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DOI:

[10.1074/jbc.M006597200](https://doi.org/10.1074/jbc.M006597200)

[10.1074/jbc.M006597200](https://doi.org/10.1074/jbc.M006597200)

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*Citation for published version (Harvard):*

da Silva Xavier, G, Varadi, A, Ainscow, EK & Rutter, GA 2000, 'Regulation of Gene Expression by Glucose in Pancreatic  $\beta$ -Cells (MIN6) via Insulin Secretion and Activation of Phosphatidylinositol 3'-Kinase', *Journal of Biological Chemistry*, vol. 275, no. 46, pp. 36269-36277. <https://doi.org/10.1074/jbc.M006597200>, <https://doi.org/10.1074/jbc.M006597200>

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Checked for eligibility: 19/06/2018

This research was originally published in the Journal of Biological Chemistry. Gabriela da Silva Xavier, Aniko Varadi, Edward K. Ainscow and Guy A. Rutter. Regulation of Gene Expression by Glucose in Pancreatic  $\beta$ -Cells (MIN6) via Insulin Secretion and Activation of Phosphatidylinositol 3'-Kinase. J. Biol. Chem. 2000; 275, 36269-36277. © the American Society for Biochemistry and Molecular Biology.

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## Regulation of Gene Expression by Glucose in Pancreatic $\beta$ -Cells (MIN6) via Insulin Secretion and Activation of Phosphatidylinositol 3'-Kinase\*

Received for publication, July 24, 2000, and in revised form, August 22, 2000  
Published, JBC Papers in Press, August 30, 2000, DOI 10.1074/jbc.M006597200

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Increases in glucose concentration control the transcription of the preproinsulin (PPI) gene and several other genes in the pancreatic islet  $\beta$ -cell. Although recent data have demonstrated that secreted insulin may regulate the PPI gene (Leibiger, I. B., Leibiger, B., Moede, T., and Berggren, P. O. (1998) *Mol. Cell* 1, 933–938), the role of insulin in the control of other  $\beta$ -cell genes is unexplored. To study the importance of insulin secretion in the regulation of the PPI and liver-type pyruvate kinase (L-PK) genes by glucose, we have used intranuclear microinjection of promoter-luciferase constructs into MIN6  $\beta$ -cells and photon-counting imaging. The activity of each promoter was increased either by 30 (versus 3) mM glucose or by 1–20 nM insulin. These effects of insulin were not due to enhanced glucose metabolism since culture with the hormone had no impact on the stimulation of increases in intracellular ATP concentration caused by 30 mM glucose. Furthermore, the islet-specific glucokinase promoter and cellular glucokinase immunoreactivity were unaffected by 30 mM glucose or 20 nM insulin. Inhibition of insulin secretion with the  $\text{Ca}^{2+}$  channel blocker verapamil, the ATP-sensitive  $\text{K}^{+}$  channel opener diazoxide, or the  $\alpha_2$ -adrenergic agonist clonidine blocked the effects of glucose on L-PK gene transcription. Similarly, 30 mM glucose failed to induce the promoter after inhibition of phosphatidylinositol 3'-kinase activity with LY294002 and the expression of dominant negative-acting phosphatidylinositol 3'-kinase ( $\Delta p85$ ) or the phosphoinositide 3'-phosphatase PTEN (phosphatase and *tensin* homologue). LY294002 also diminished the activation of the L-PK gene caused by inhibition of 5'-AMP-activated protein kinase with anti-5'-AMP-activated protein kinase  $\alpha 2$  antibodies. Conversely, stimulation of insulin secretion with 13 mM KCl or 10  $\mu\text{M}$  tolbutamide strongly activated the PPI and L-PK promoters. These data indicate that, in MIN6  $\beta$ -cells, stimulation of insulin secretion is important for the activation by glucose of L-PK as well as the PPI promoter, but does not cause increases in glucokinase gene expression or glucose metabolism.

Elevated glucose concentrations stimulate the transcription

of the preproinsulin (PPI)<sup>1</sup> gene (1, 2) and several other genes in islet  $\beta$ -cells, including the liver-type pyruvate kinase (L-PK) gene (3), acetyl-CoA carboxylase I (4), GLUT2 (5), and a raft of other genes involved in insulin synthesis and release (6). However, the molecular mechanisms involved in the regulation of transcription by glucose are only partly understood (7, 8). Recent observations have suggested that the release of insulin may play an important part in the regulation of the preproinsulin gene by glucose, at least under certain conditions (9, 10). Consistent with this, increases in intracellular free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ), which are important in the activation of insulin release, have been reported to be essential for regulation of PPI gene expression by glucose in some systems (11, 12). On the other hand, several studies have indicated that glucose can also regulate the PPI gene independently of insulin secretion (13–15). Furthermore, PPI gene expression appears to be largely unaltered after targeted disruption of the insulin receptor gene in the islet  $\beta$ -cell, at least in younger animals (16).

By contrast, the role of insulin secretion in the regulation of other glucose-sensitive islet  $\beta$ -cell genes is largely uninvestigated (8, 17). Elevations of glucose concentration enhance the expression of the L-PK gene in hepatocytes through activated transcription (18). This effect of glucose is dependent upon a *cis*-acting upstream region of the gene from nucleotides –183 to +10 with respect to the cap site (19), and a glucose response element has been mapped to a palindromic repeat of two E-boxes (CACGGG) located in the region at base pairs –170 to –150 with respect to the transcriptional start site (20). A similar region is also present in other glucose-responsive genes (8), including those encoding Spot14 (21), acetyl-CoA carboxylase I (22, 23), and fatty-acid synthase (24).

Phosphorylation of glucose appears to be essential for the transcriptional effects of the sugar on L-PK gene transcription in liver (7). Thus, glucose 6-phosphate and the pentose phosphate intermediate xylulose 5-phosphate (25) may be key signaling intermediates. The identity of the transcription factors mediating the transcriptional response is still debated, with evidence both for (26, 27) and against (28, 29) a role for the ubiquitous upstream stimulatory factor (USF1 and USF2). Recent data have also implicated sterol response element-binding protein-1c (SREBP-1c) (30) and other less well defined factors (31).

L-PK gene transcription is also strongly stimulated by insu-

\* This work was supported by project grants from the Wellcome Trust, Diabetes UK (formerly the British Diabetes Association), the Medical Research Council (United Kingdom), and the Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: PPI, preproinsulin; L-PK, liver-type pyruvate kinase;  $[\text{Ca}^{2+}]_i$ , intracellular free  $\text{Ca}^{2+}$  concentration; USF, upstream stimulatory factor; SREBP-1c, sterol response element-binding protein-1c; GK, glucokinase (hexokinase type IV); PI3K, phosphatidylinositol 3'-kinase; AMPK, 5'-AMP-activated protein kinase; KRB, Krebs-Ringer bicarbonate; CMV, cytomegalovirus; PIP<sub>3</sub>, phosphatidylinositol 3,4,5-trisphosphate.

lin in cultured liver cells. Under most experimental conditions, this effect requires elevated glucose concentrations and has been considered to be due mainly to up-regulation of the glucokinase (GK) gene (32) and thus increased intracellular concentrations of Glu-6-P or xylulose 5-phosphate (33, 34). However, insulin also activates the L-PK promoter after culture of hepatocytes from starved rats after constitutive expression of GK (35), indicating that an additional, glucose-independent mechanism of action of the hormone must exist.

Since high glucose concentrations stimulate islet GK gene expression relatively weakly (36, 37), if at all (3, 32), the inductive effect of glucose on L-PK gene transcription in islets and  $\beta$ -cell-derived INS-1 (3) and MIN6 (26, 38) cells has previously been attributed solely to an increase in the intracellular concentration of a glucose metabolite (e.g. Glu-6-P or xylulose 5-phosphate; see above). However, glucose also stimulates the release of stored insulin from the  $\beta$ -cell, so the potential exists for a para- or autocrine effect of the hormone on gene expression. We have therefore investigated the effects of added insulin on the PPI, L-PK, and GK gene promoters in the highly glucose-responsive model MIN6  $\beta$ -cell line (39). This system has enabled us to determine the relative importance of activated insulin secretion, glucose metabolism, and changes in  $[Ca^{2+}]_i$  in the transcriptional regulation of the L-PK gene by glucose. Our results suggest that, under appropriate conditions, secretion of insulin and the activation of a signaling pathway dependent upon phosphatidylinositol 3'-kinase (PI3K) largely explain the transcriptional effects of glucose on PPI and L-PK gene expression in this  $\beta$ -cell model.

#### EXPERIMENTAL PROCEDURES

**Materials**—Dulbecco's modified Eagle's medium was from Sigma (Dorset, United Kingdom) or Life Technologies, Inc. (Paisley, UK). Beetle luciferin was from Promega, and coelenterazine was from Molecular Probes, Inc. Polyclonal anti-glucokinase antibody (sc-7908) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). LY294002 was from Calbiochem-Novabiochem (Nottingham, UK). Porcine insulin was from Sigma. Other reagents were from Sigma or BDH.

**Plasmids**—pLPK-Luc<sub>FF</sub> and p(-150)LPK-Luc<sub>FF</sub> contained nucleotides -183 to +10 and -148 to +10, respectively, of the rat L-PK promoter fused immediately upstream of humanized firefly luciferase cDNA (Promega plasmid pGL3-Basic<sup>TM</sup>). Plasmid pINS-Luc<sub>FF</sub> contained nucleotides -260 to -60 of the human insulin promoter fused upstream of a minimal herpes simplex thymidine kinase promoter and luciferase cDNA (40). The expression plasmid for *Renilla* luciferase (pRL-CMV) was purchased from Promega. pSR $\alpha$ Ap85 (provided by Prof. J. Tavaré) was used to express, under SV40 promoter control, a dominant negative-acting mutant of the p85 subunit of PI3K that lacks the binding site for the catalytic p110 subunit of PI3K (41). Plasmid pCMV5-PTEN was supplied by Prof. J. E. Dixon (University of Michigan). Plasmid  $\beta$ GK4-Luc, containing region -4000 to +1 of the rat  $\beta$ -cell glucokinase promoter (3), was provided by Dr. P. Iynedjian (University of Geneva, Geneva, Switzerland).

**Antibodies**—Affinity-purified sheep antibodies raised against rat 5'-AMP-activated protein kinase (AMPK)  $\alpha$ 2 (42) were provided by Prof. D. G. Hardie (University of Dundee, Dundee, Scotland) and extensively dialyzed against microinjection buffer (2 mM Tris-HCl (pH 8.0) and 0.2 mM Na<sup>+</sup> EDTA) before use.

**Cell Culture**—MIN6  $\beta$ -cells (39) were used between passages 19 and 25 and grown in Dulbecco's modified Eagle's medium containing 15% (v/v) heat-inactivated fetal calf serum, 25 mM glucose, 5.4 mM KCl, 2 mM glutamine, 100 mM  $\beta$ -mercaptoethanol, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin in a humidified atmosphere at 37 °C with 5% CO<sub>2</sub> unless specified otherwise. Cells were transferred to medium containing 3 mM glucose 16 h prior to experiments. Secreted insulin was measured by radioimmunoassay (Linco Research Inc., St. Charles, MO) (43).

**Infection with Adenoviruses**—Cells were exposed to adenoviruses (30–100 viral particles/cell) for 2 h prior to culture for a further 24 h in the absence of added virus (43).

**Microinjection and Luciferase Imaging**—Intranuclear microinjection of plasmids and antibodies was performed using an Eppendorf 5121/5246 micromanipulator (26, 44) at plasmid concentrations of 0.1 (Luc<sub>FF</sub>-

based vectors) and 0.05 (pCMV-RL) mg/ml and a total antibody concentration of 1–1.5 mg/ml (Bradford assay (45)) in 2 mM Tris-HCl, pH 8.0, 0.2 mM EDTA (38). Plasmid pSR $\alpha$ Ap85 or the corresponding empty vector (p85SR $\alpha$ ) was injected at 0.1 mg/ml. Individual experiments involved injection of 100–200 separate cells/condition, with an efficiency of 5–20% productive injection as assessed by expression of *Renilla reniformis* luciferase activity. Cells were imaged 6 h after microinjection and cultured under the conditions described above. Photon-counting imaging of firefly and *R. reniformis* luciferase activities was performed in single living cells using an Olympus IX-70 inverted microscope ( $\times$ 10 air objective, 0.4 NA) and an intensified charge-coupled device camera (Photek, East Sussex, UK) as described in detail previously (26, 46).

**Measurement of  $[Ca^{2+}]_i$  and Insulin Secretion**—Changes in  $[Ca^{2+}]_i$  were measured at 37 °C with entrapped fura-2 (43, 47) using a Leica DM-IRBI inverted microscope ( $\times$ 40 objective) and a Hamamatsu C4742-995 charge-coupled device camera driven by OpenLab<sup>TM</sup> software (Improvision, Coventry, UK) (43, 48). Cells were loaded for 40 min in modified Krebs-Ringer bicarbonate (KRB) buffer (125 mM NaCl, 3.5 mM KCl, 1.5 mM CaCl<sub>2</sub>, 0.5 mM MgSO<sub>4</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM NaHCO<sub>3</sub>, and 10 mM Na<sup>+</sup>-Hepes (pH 7.4) initially containing 3 mM glucose and equilibrated with 95:5 O<sub>2</sub>/CO<sub>2</sub>) supplemented with 5  $\mu$ M fura-2/AM (Sigma) and 0.1% Pluronic F-127 (BASF, Mount Olive, NJ). Insulin released into the culture medium was assayed by radioimmunoassay (Linco Research Inc.) (43).

**Intracellular ATP Concentration Imaging with Recombinant Expressed Luciferase**—Cytosolic luciferase (49) was expressed using an adenoviral vector (50) under constitutive (CMV) promoter control (*Ad-CMVcLuc*)<sup>2</sup> and monitored by real-time photon-counting imaging during perfusion in KRB buffer (37 °C) (43, 49).

**Statistical Analysis**—Data are given as means  $\pm$  S.E. of three to five individual experiments. Comparisons between means were performed using one-tailed Student's *t* test for paired data with Microsoft Excel<sup>TM</sup>.

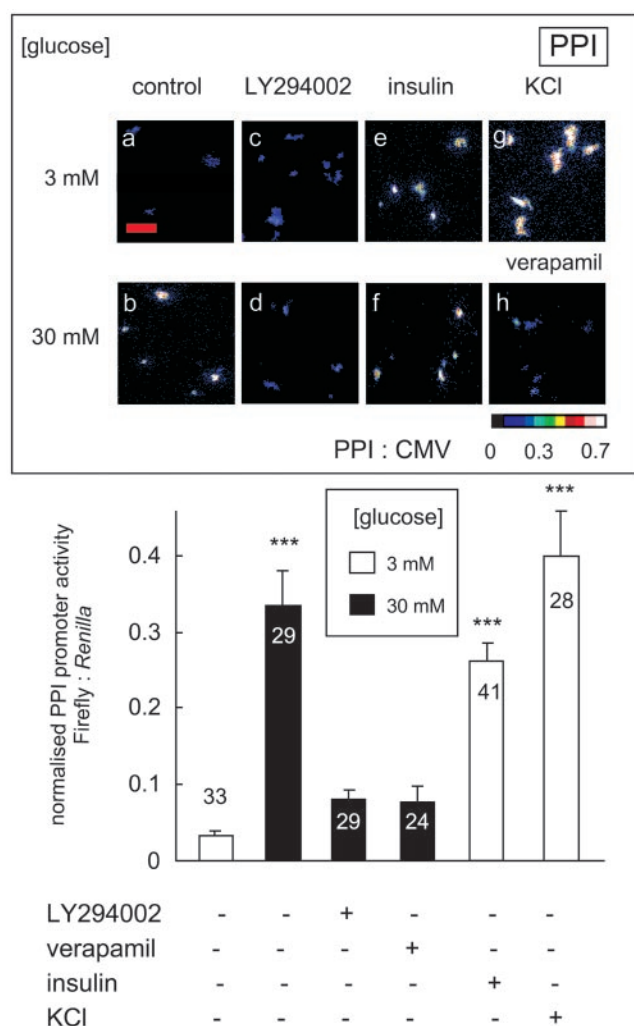
#### RESULTS

**Regulation of the PPI Promoter by Glucose and Exogenous Insulin in Single MIN6  $\beta$ -Cells**—The activity of the human PPI promoter (base pairs -260 to -60 with respect to the transcriptional start site) (40) was measured by single cell luminescence assay (46) after microinjection of a firefly luciferase reporter construct. This promoter region contains the homeodomain-binding elements A1, A2, and A3, as well as the E-boxes E1 and E2 (51). PPI promoter activity was normalized to that of the CMV immediate-early gene promoter, linked to the expression of *R. reniformis* luciferase (26, 46, 52). In a previous report (14), we described conditions under which the PPI promoter was regulated in single MIN6  $\beta$ -cells by glucose, independently of secreted insulin. In the present study, we observed that, by culturing MIN6 cells at (a) a lower passage number (passages 19–25) and (b) a lower density (thus decreasing the contribution of secreted insulin at low glucose concentrations), the PPI promoter was up-regulated more strongly (5–10-fold versus 1.5–2-fold in our previous report (14)) by 30 mM glucose and to a similar extent by 20 nM insulin (Fig. 1). The effect of 30 mM glucose was strongly inhibited by the L-type Ca<sup>2+</sup> channel inhibitor verapamil and the type I PI3K inhibitor LY294002 (38). The effects of 30 mM glucose were also mimicked by low concentrations of KCl (13 mM) (Fig. 1), as previously reported for islet  $\beta$ -cells and HIT-T15 cells (9). However, higher KCl (>30 mM) and insulin (100 nM) concentrations were ineffective (data not shown), as described in previous MIN6 cultures (49).

**Regulation of the L-PK Promoter by Glucose and Exogenous Insulin**—In MIN6 cells co-microinjected with a minimal L-PK promoter (base pairs -183 to +10)-firefly luciferase reporter construct (26) and maintained for 6 h at 3 mM glucose, the ratio of firefly to *Renilla* luciferase activity was in the range 0.02–0.15, with higher values associated with later cell passage numbers. In common with previous studies (26, 52), 30 mM glucose stimulated the L-PK promoter by  $3.6 \pm 0.07$ -fold (11

<sup>2</sup> G. A. Rutter and E. K. Ainscow, submitted for publication.





**FIG. 1. Regulation of PPI promoter activity by glucose and insulin.** MIN6 cells were microinjected with plasmids pPPI-Luc<sub>FF</sub> (0.1 mg/ml) and pCMV-RL (0.05 mg/ml) as described under "Experimental Procedures" before culture for 6 h at the indicated glucose concentrations with the following additions: none (upper panel, panels a and b), 50  $\mu$ M LY294002 (panels c and d), 20 nM insulin (panels e and f), 13 mM KCl (final concentration; panel g), and 100  $\mu$ M verapamil (panel h). Normalized PPI promoter activity (obtained from the ratio of firefly to *Renilla* luciferase activities; pseudo-color) was determined after photon-counting imaging in the presence of luciferin and coelenterazine *n*. Shown are typical regions ( $\sim 0.06$  mm<sup>2</sup>) from a total injected area of  $\sim 0.25$  mm<sup>2</sup>. Scale bar = 50  $\mu$ m. The pseudo-color bar represents the ratio of firefly luciferase (reporting PPI promoter activity) to *Renilla* luciferase activity (CMV promoter). Combined data (means  $\pm$  S.E.) from three separate experiments are shown in the lower panel and report observations on the total number of individual cells given on the bars. \*\*\*,  $p < 0.001$  for the effect of glucose, insulin, or KCl.

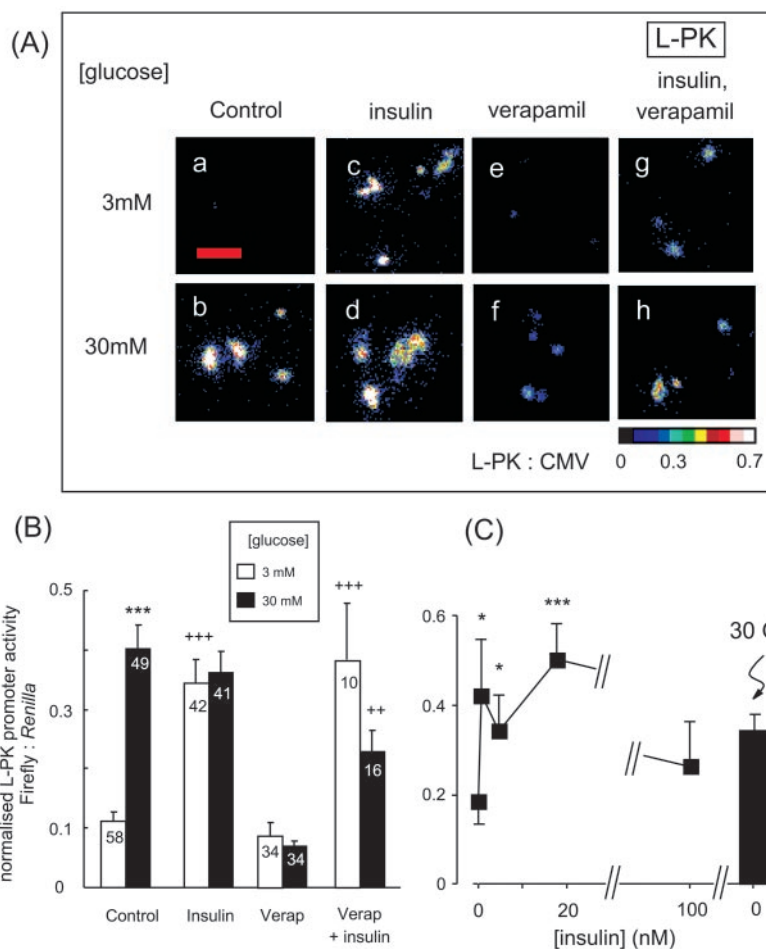
separate experiments,  $n = 108$  and 126 cells incubated at 3 and 30 mM glucose, respectively;  $p < 0.001$  for the effect of 30 mM glucose) (Fig. 2, A, panel b; and B). This effect was mimicked by the addition of exogenous insulin (20 nM). Thus, in 11 experiments on separate cultures, 20 nM insulin increased the activity of the L-PK promoter in cells maintained at 3 mM glucose by  $3.3 \pm 0.002$ -fold ( $n = 108$  control and 67 insulin-stimulated cells;  $p < 0.001$ ) (Fig. 2, A, panel c; and B). By contrast, added insulin was entirely without effect at stimulatory glucose concentrations (30 mM) (Fig. 2, A, panels b and d; and B). At 3 mM glucose, the effect of insulin was clearly apparent at low physiological concentrations of the hormone, with 1 nM insulin providing  $>50\%$  maximal stimulation of promoter activity (Fig. 2C). By contrast, 100 nM insulin failed to stimulate L-PK promoter activity significantly (Fig. 2C).

To determine whether secretion of insulin may be responsible for the increase in L-PK promoter activity at high glucose concentration, we measured the concentrations of insulin present in the incubation medium at the end of the culture period. After 6 h of incubation at 3 and 30 mM glucose, the medium insulin concentrations were  $7.08 \pm 2.25$  and  $13.3 \pm 4.25$  nM, respectively ( $n =$  three separate cultures). Thus, elevated glucose stimulated insulin secretion in the cell cultures as expected and over a range to which the L-PK promoter was sensitive (Fig. 2C). We next tested the effect of blocking the exocytosis of insulin in response to glucose with verapamil. In the presence of this inhibitor, 30 mM glucose had no effect on L-PK promoter activity (Fig. 2, A, panels e and f; and B). However, in the presence verapamil, the addition of 20 nM insulin still caused a marked activation of L-PK promoter activity at 3 or 30 mM glucose (Fig. 2, A, panels e–h; and B), demonstrating that the effects of verapamil are unlikely to be due to any nonspecific toxic effects of the drug. Similarly, diazoxide, which opens the ATP-sensitive  $K^+$  channel (53) and thus prevents cell depolarization and  $[Ca^{2+}]_i$  increases (Fig. 3c), also eliminated induction of the L-PK promoter with 30 mM glucose (Fig. 3a). To determine whether the effects of diazoxide or verapamil may be due to an inhibition of  $Ca^{2+}$ -dependent glucose metabolism (49, 54), we used adenovirally expressed firefly luciferase,<sup>2</sup> under the control of the strong cytomegalovirus immediate-early gene promoter, to monitor the free cytoplasmic ATP concentration in real time (49). Perfusion of cell populations with 30 mM glucose led to a clear and rapid increase in ATP-dependent luciferase bioluminescence ( $\sim 15\%$  above basal levels), which was unaffected by the presence of 200  $\mu$ M diazoxide (Fig. 3d). Finally, inhibition of insulin secretion with the  $\alpha_2$ -adrenergic agonist clonidine (1  $\mu$ M), which has no effect on glucose-induced  $[Ca^{2+}]_i$  or [ATP] increases (data not shown),<sup>3</sup> also strongly inhibited the effect of glucose on the L-PK promoter (Fig. 3b). Clonidine had no impact on the ability of insulin (20 nM) to stimulate the L-PK promoter (Fig. 3b).

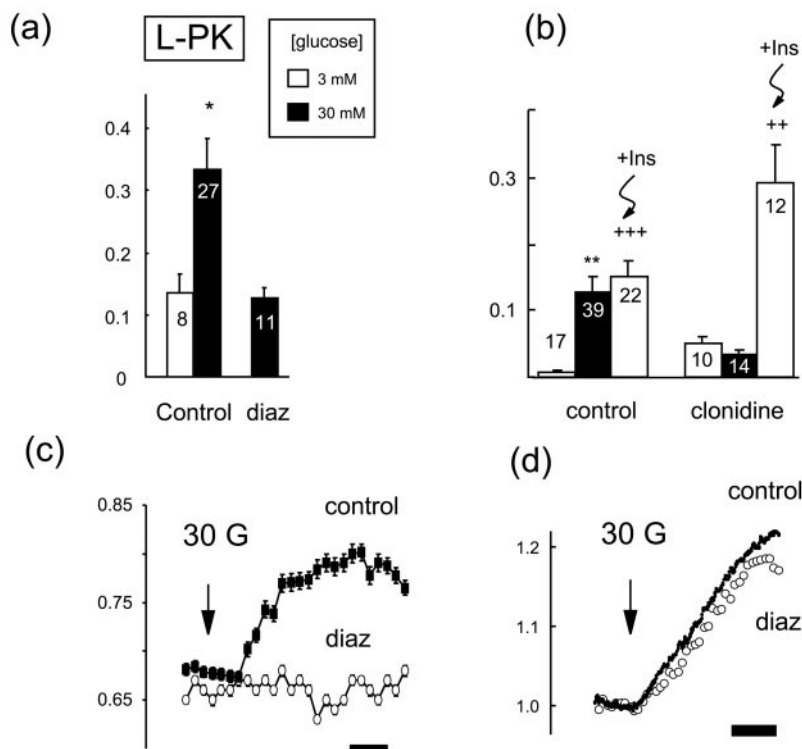
To test whether the activation of insulin secretion may be sufficient, in the absence of a nutrient stimulus, to activate the L-PK promoter, we next examined the effects of low concentrations of non-nutrient secretagogues. Increasing the concentration of KCl in the culture medium from 5.4 to 13 mM or adding 10  $\mu$ M tolbutamide stimulated L-PK promoter activity strongly (Fig. 4, a and c). Measured in perfused cells, each agent caused a clear, but relatively small increase in  $[Ca^{2+}]_i$  (Fig. 4, b and d). Importantly, the increase in  $[Ca^{2+}]_i$  elicited by the lower concentrations of KCl and tolbutamide resembled closely that evoked by 30 mM glucose (an increase in fura-2 emission ratio of  $\sim 0.15$  arbitrary ratio units). The addition to the medium of higher concentrations of either agent (30 mM KCl or 200  $\mu$ M tolbutamide) stimulated the L-PK promoter much more weakly (Fig. 4, a and c) while causing much larger increases in  $[Ca^{2+}]_i$  (Fig. 4, b and d) and insulin secretion (data not shown). However, a clear decrease in cell viability and cell morphology was apparent after 6 h of incubation with these agents (data not shown).

**Effect of PI3K Inhibition on the Activation by Glucose of the L-PK Promoter**—We next examined the dependence of the effects of glucose on activation of PI3K. The PI3K inhibitor LY294002 completely abolished the effect of 30 mM glucose (Fig. 5a). Indeed, under these conditions, L-PK promoter activity was significantly inhibited at 30 mM glucose compared with 3 mM glucose. To determine whether the inhibitory effect of high glucose may be due to an (undefined) response element lying 3' to the L-PK glucose response element, we also examined the effects of glucose on the activity of a truncated L-PK promoter bearing only nucleotides  $-148$  to  $+10$ . This promoter

**FIG. 2. Effect of glucose and insulin on the L-PK gene promoter in single MIN6  $\beta$ -cells.** A, cells were microinjected with plasmids pLPK-Luc<sub>FF</sub> (0.1 mg/ml) and pCMV-RL (0.05 mg/ml) as described under "Experimental Procedures" before culture for 6 h at the indicated glucose concentrations with the following additions: none (panels a and b), 20 nM insulin (panels c and d), 100  $\mu$ M verapamil (panels e and f), and 100  $\mu$ M verapamil plus 20 nM insulin (panels g and h). Photon production by firefly (reporting L-PK promoter activity) and *Renilla* (reporting CMV promoter activity) luciferases was quantitated as described under "Experimental Procedures," and the ratio of the two was calculated to provide a normalized assay of L-PK promoter function (pseudo-color). Scale bar = 50  $\mu$ m. B, data were from two to five separate experiments as shown in A, involving the number of cells given. Verap, verapamil. C, shown is the dose response for added insulin (3 mM glucose). Data were from 10–70 cells for each insulin concentration. \*\*\* and \*,  $p < 0.001$  and  $p < 0.05$  for the effect of 30 mM glucose (30 G), respectively; +++ and ++,  $p < 0.001$  and  $p < 0.01$  for the effect of insulin, respectively.



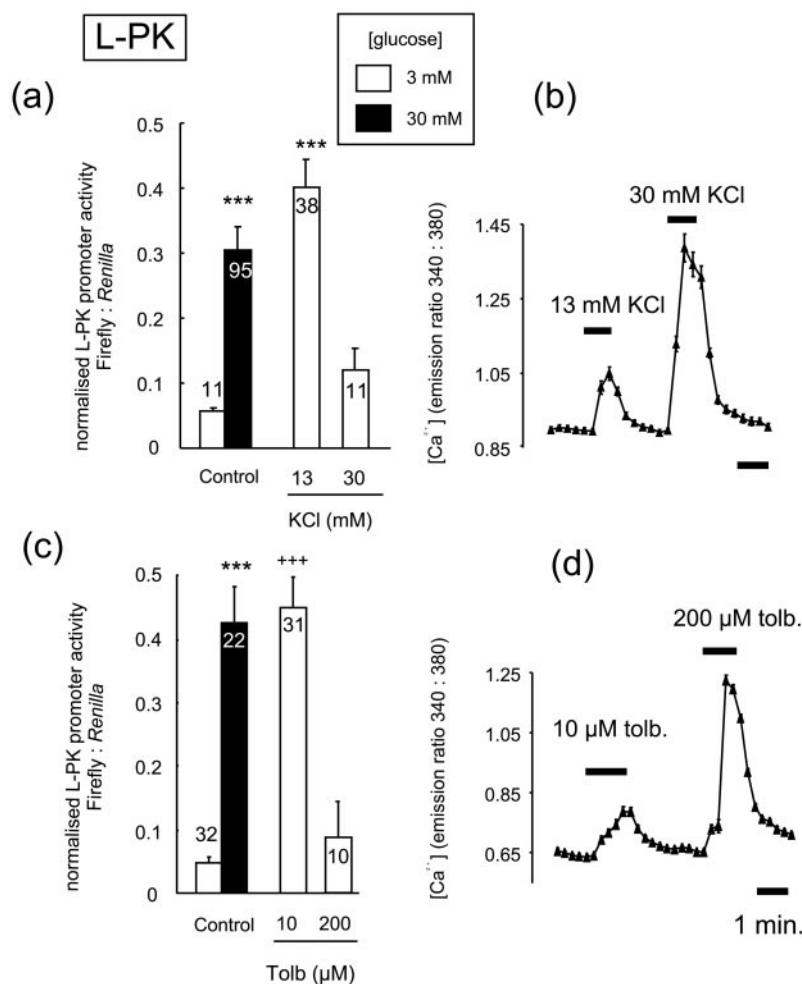
**FIG. 3. Effect of inhibiting insulin secretion on L-PK promoter activity.** a and b, microinjection and imaging were performed as described in the legend to Fig. 2, with additions of diazoxide (diaz; 200  $\mu$ M) and clonidine (1  $\mu$ M) as indicated. The number of injected cells (from three separate cultures) is given on the bars. \*\* and \*,  $p < 0.01$  and  $p < 0.05$  for the effect of glucose, respectively; ++ and +,  $p < 0.01$  and  $p < 0.05$  for the effect of 20 nM insulin (Ins), respectively. c, changes in  $[Ca^{2+}]_i$  in response to an increase in peripartate glucose concentration from 3 to 30 mM glucose (30 G) were measured in fura-2-loaded cells. Data were from experiments on four separate cultures and are the means  $\pm$  S.E. of values from 49 control cells or 50 cells preincubated and perfused in the presence of diazoxide. d, cytosolic [ATP] was measured in AdCMV-Luc-transduced cells as described under "Experimental Procedures." Shown are typical traces from three separate experiments. The presence of diazoxide (added 5 min prior to 30 mM glucose) in c and d is indicated by the open symbols. The time bars represent 60 s.



was also significantly inhibited by 30 versus 3 mM glucose (Fig. 5a). Confirming that the effects of LY294002 were likely to be specific for PI3K, overexpression of a dominant-negative form

of the PI3K adaptor subunit ( $\Delta p85$ ) lacking the domain responsible for binding the catalytic subunit of PI3K (p110) (38, 41) led to activation of the L-PK promoter in cells maintained at 3

**FIG. 4. Effect of glucose-independent activation of insulin secretion on L-PK promoter activity.** Microinjection, luciferase imaging, and measurements of  $[Ca^{2+}]_i$  were performed as described in the legend to Fig. 2. Cells were incubated in culture medium at the indicated glucose and KCl concentrations (a and c) or perfused in KRB buffer at 3 mM glucose (b and d). In all cases,  $[KCl]$  was 5.4 mM unless otherwise indicated. *Tolb* and *tolb.*, tolbutamide. Data were from experiments on four to five separate cultures involving the number of individual cells shown on the bars (a and c) or 52 (b) and 42 (d) separate cells. The time bars indicate 60 s.



mM glucose, but a powerful inhibition of promoter activity in cells maintained at 30 mM glucose (Fig. 5b).

To determine whether the inositol phospholipid or protein kinase activities of PI3K (55) mediated the effects of glucose and insulin on L-PK promoter activity, we examined the effects of overexpressing the inositol-3'-phosphate phosphatase PTEN (phosphatase and *tensin* homologue) (56). PTEN expression completely ablated the activation of the L-PK promoter by glucose (Fig. 5c).

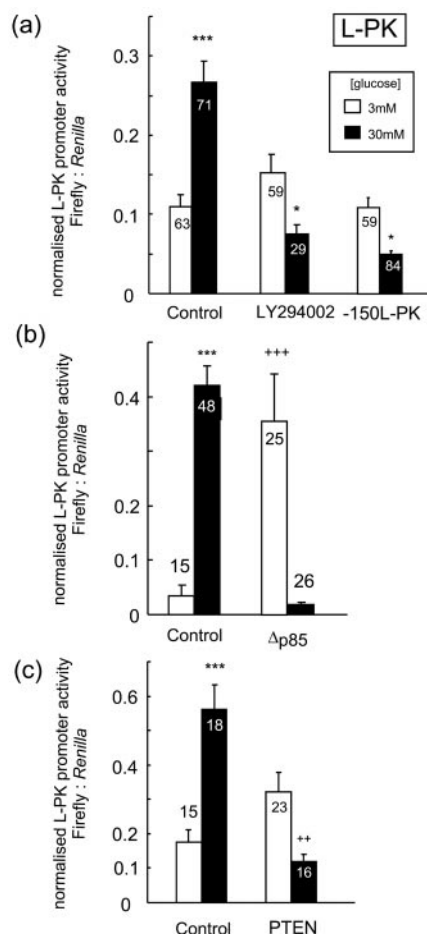
**Effects of Glucose and Insulin on Glucokinase Promoter Activity**—Adenovirally mediated overexpression of liver glucokinase (57, 58) strongly increased L-PK promoter activity both at 3 mM glucose (from a firefly/*R. reniformis* luminescence ratio of 0.008 ( $n = 12$  null virus-infected cells) to 0.196 ( $n = 16$  GK virus-infected cells)) and at 30 mM glucose (from 0.129 ( $n = 39$  null cells) to 0.241 ( $n = 9$  infected cells)). However, at 3 mM glucose, insulin (20 nM) still elicited a further induction of L-PK promoter activity after the overexpression of GK (firefly/*R. reniformis* luminescence ratio of  $0.73 \pm 0.08$ ,  $n =$  six insulin-treated cells). Furthermore, neither 30 mM glucose nor exogenous insulin (20 nM) affected the activity of a reporter construct encoding 4 kilobase pairs of the islet-specific glucokinase promoter (Fig. 6a) (59). Finally, immunocytochemical analysis revealed no detectable change in the level of glucokinase protein after 6 h culture at 3 or 30 mM glucose or at 3 mM glucose plus insulin (data not shown).

**Effect of Glucose and Insulin on Intracellular Free ATP Concentration**—To examine further whether the effects of insulin or elevated glucose may be due to an enhancement of glucose metabolism independent of glucokinase gene expression, we

used adenovirally expressed firefly luciferase<sup>2</sup> as described above (49). Demonstrating that this assay reported increases in glycolytic flux, overexpression of glucokinase with the adenoviral vector dramatically altered the responses of intracellular [ATP] to changes in glucose concentration (Fig. 6b). In control (null virus-infected) cells, increases in apparent cellular free [ATP] were not evident until the glucose concentration was raised to 30 mM (Fig. 6b, *open circles*) and were substantially blocked by the GK inhibitor mannoheptulose (data not shown). By contrast, in GK-overexpressing cells, intracellular free [ATP] was increased considerably by 3 mM glucose, but lowered when glucose was further raised to 30 mM (Fig. 6b, *closed circles*). This drop presumably reflects ATP consumption by the glucokinase reaction itself under conditions in which distal steps in glycolysis or glucose oxidation exert a significant control strength (58). The increase in luminescence of 15–20% seen in control cells in response to 30 mM glucose (Figs. 3, d; and 6, b–d) was unaffected by culture in the presence of insulin for 6 h (Fig. 6c) or after acute (5 min) exposure to the hormone (Fig. 6d). These experiments also reveal that the contribution of changes in [ATP] to the changes in the apparent promoter activity reported with firefly luciferase constructs are unlikely to exceed 20% (and have therefore been ignored).

**Role of PI3K in the Effects of AMPK- $\alpha$ 2**—We have previously demonstrated that inhibition of the  $\alpha$ 2 form of AMPK activates the L-PK promoter, mimicking the effect of glucose (52). To determine whether AMPK lies upstream or downstream of PI3K, we examined the effect of PI3K inhibition when AMPK activity had been inhibited by microinjection of anti-AMPK- $\alpha$ 2 antibodies (52) (Fig. 7). The stimulatory effect of inhibiting





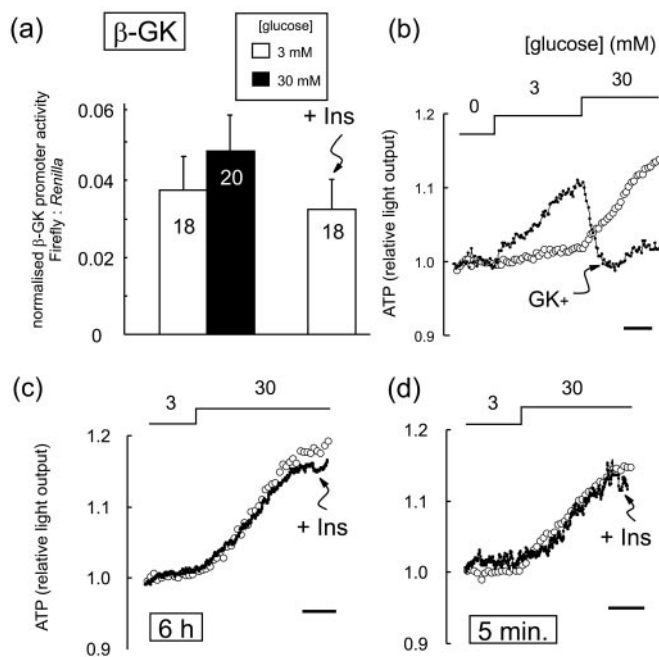
**FIG. 5. Effect of inhibiting PI3K function on L-PK promoter activity.** *a*, MIN6 cells were microinjected as described under “Experimental Procedures” with plasmids pCMV-RL and pLPK-Luc<sub>FF</sub> or p(-150)LPK-Luc<sub>FF</sub> (-150L-PK) (as indicated) before 6 h of culture at 3 or 30 mM glucose as indicated in the absence or presence of 50  $\mu$ M LY294002. \*\*\* and \*,  $p < 0.001$  and  $p < 0.05$  for the effect of glucose, respectively. *b*, same as *a*, but with co-microinjection of the plasmid encoding  $\Delta p85$  or the corresponding empty vector (pSR $\alpha$ ; Control). \*\*\* and +,  $p < 0.001$  for the effect of 30 mM glucose and LY294002, respectively. *c*, same as *a*, but after co-injection of the expression plasmid encoding PTEN or the empty vector (pCMV5; Control) as indicated. ++,  $p < 0.01$  for the effect of PTEN expression relative to the control (30 mM glucose).

AMPK- $\alpha 2$  activity was also strongly reversed by inhibition of PI3K with LY294002 (Fig. 7).

#### DISCUSSION

**Role of Insulin Secretion in the Regulation of the PPI Gene by Glucose**—This study demonstrates that, in the highly glucose-responsive MIN6  $\beta$ -cell line, stimulation of exocytosis by glucose plays a key role in activating the L-PK gene as well as PPI gene expression. It should be stressed that this study was performed under conditions different from those of our recent report (14), in which glucose was able to activate the PPI promoter in MIN6 cells relatively weakly ( $\sim 1.5$ -fold) and independently of insulin secretion. Although not investigated in the present study, it seems likely that changes in the distribution (38, 40) or DNA-binding activity (60) of the homeodomain A3-binding protein PDX-1 (pancreatic duodenum homeobox-1; formerly termed IPF-1, IDX-1, STF-1, and IUF-1) (61) are likely to play an important role in this activation.

**Comparison of the Role of Insulin Secretion in the Regulation of PPI and L-PK Genes by Glucose**—In this report, we demonstrate that the L-PK gene, in common with the PPI gene, can be



**FIG. 6. Impact of insulin and glucose on glucokinase promoter activity and glucose metabolism in single cells.** *a*, cells were microinjected with plasmids  $\beta$ GK4-Luc (0.1 mg/ml) and pRL-CMV (0.05 mg/ml) and incubated for 6 h at the indicated glucose concentrations or at 3 mM glucose in the presence of 20 nM insulin (Ins). *b–d*, shown is the response of cytoplasmic [ATP] in MIN6 cells to 30 mM glucose. *b*, cells were transduced with adenoviral luciferase (AdCMVluc; see “Experimental Procedures”) plus adenovirus expressing GK (closed circles; GK+) or empty virus (open circles). 16 h after infection, the cells were preincubated for 1 h in KRB buffer at 0 mM glucose before perfusion in the presence of 5  $\mu$ M luciferin for detection of luciferase light emission (see “Experimental Procedures”) at the indicated glucose concentrations. *c*, cells expressing AdCMVluc alone were incubated for 6 h in culture medium containing 3 mM glucose and either 20 nM insulin (closed circles) or no insulin (open circles), and the response of [ATP] to a change in glucose from 3 to 30 mM glucose was measured as shown. *d*, cells were incubated for 16 h in medium containing 3 mM glucose and then subsequently perfused with KRB buffer supplemented with 3 mM glucose alone (open circles) or 3 mM glucose and 20 nM insulin (closed circles). After 5 min of pre-perfusion, the glucose concentration was then changed to 30 mM as shown ( $\pm 20$  nM insulin as appropriate). The time bars represent 100 s.

regulated largely by glucose through the stimulation of insulin secretion. Thus, we have used three approaches to distinguish between the roles of 1) insulin secretion, 2) changes in  $[Ca^{2+}]_i$ , and 3) glucose metabolism in the regulation of transcription. It should be emphasized that our measurements of  $[Ca^{2+}]_i$  and intracellular free [ATP] (Figs. 3, 4, and 6) were made only over the short term (minutes) compared with the longer incubations (hours) used in measurements of gene expression. These should therefore only be taken as a guide to the changes in these parameters during the full time course of measurements of gene expression (6 h). Nevertheless, the former measurements were considered important since several maneuvers that block secretion (e.g. by preventing increases in  $[Ca^{2+}]_i$ ) can also partially inhibit glucose metabolism and thus the generation of potential intracellular signaling molecules (e.g. Glu-6-P or xylulose 5-phosphate.<sup>3</sup> For example, verapamil, which completely suppressed the induction of PPI and L-PK promoter activities by 30 mM glucose (Figs. 1 and 2), also suppresses increases in  $[Ca^{2+}]_i$  and intracellular [ATP].<sup>3</sup> On the other hand, diazoxide, which blocked insulin secretion and the increase in  $[Ca^{2+}]_i$  (Fig. 3c), but not glucose metabolism (Fig. 3d), was also efficient in blocking L-PK promoter induction (Fig. 3a). Finally, clonidine, which blocks insulin secretion, but has no effect on glucose metabolism and little effect on  $[Ca^{2+}]_i$ ,<sup>3</sup> was also a

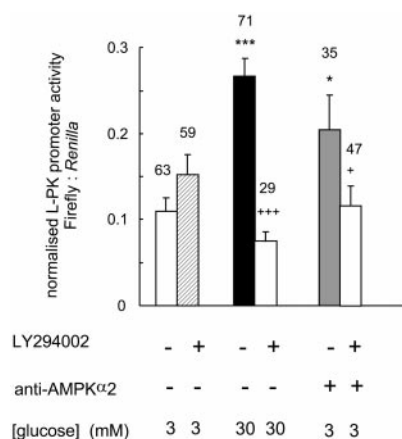


FIG. 7. **Effect of LY294002 on the stimulation of L-PK promoter activity by anti-AMPK- $\alpha$ 2 antibodies.** Cells were microinjected with plasmids pLPK-Luc<sub>FF</sub> and pCMV-RL and with control IgG or anti-AMPK- $\alpha$ 2 antibodies (1.5 mg/ml) and incubated at the indicated glucose concentrations in the presence or absence of 50  $\mu$ M LY294002 (hatched bars). \*\*\* and \*,  $p < 0.001$  and  $p < 0.05$  for the effect of 30 mM glucose, respectively; +++ and +,  $p < 0.001$  and  $p < 0.05$  for the effect of LY294002, respectively. Data in the first through fourth bars are transposed from Fig. 5 for comparison.

potent inhibitor of L-PK promoter induction (Fig. 3b). Together, these data suggest that insulin secretion, but not an increase in glucose metabolism or increases in  $[Ca^{2+}]_i$ , is the chief trigger for L-PK gene activation by glucose in the MIN6  $\beta$ -cell model.

The induction of the PPI promoter by insulin reported here is consistent with recent reports (9) and with the effects of a range of nutrient secretagogues in HIT-T15 cells (15) and MIN6 cells (60). Strikingly, higher concentrations of either KCl or tolbutamide exerted no stimulatory effect on the apparent transcriptional activity of either the PPI or L-PK gene or were inhibitory (Fig. 4, a and c). We suspect that this may be due to toxic effects of sustained high  $Ca^{2+}$  levels in the  $\beta$ -cell type, *i.e.* the positive effect of the released insulin may be counteracted by the negative influence of high  $[Ca^{2+}]_i$ . It should be noted that the stimulatory effects of higher concentrations of KCl (50 mM) or tolbutamide (100  $\mu$ M) on PPI mRNA levels, observed in previous studies (9), involved cell stimulation for only 5 min and extraction of RNA after 90 min. The absence of any effect of higher concentrations (100 nM) of insulin on L-PK promoter activity (Fig. 2C) in the present work may be related to increases in  $[Ca^{2+}]_i$  caused by added insulin itself (62)<sup>3</sup> and thus to deleterious effects on cell function. These may become particularly pronounced over longer periods of culture and may explain the absence of stimulation by sulfonylurea seen after culture of islets for 48 h by de Vargas *et al.* (13). More difficult to explain, however, is the absence of a negative effect of either nifedipine or verapamil in these earlier studies and in those of Goodison *et al.* (15) in HIT-T15 cells. Whether these agents remain stable over very extended periods (>24 h) of culture is, however, uncertain; metabolism of verapamil *in vivo* has been described (63).

**Role of PI3K in the Regulation of L-PK and PPI Genes**—A number of recent studies (9, 38, 60, 64) have implicated a role for PI3K in the regulation of the PPI promoter. The involvement of PI3K activity in the regulation of the L-PK promoter was demonstrated in these studies through the effects of LY294002 and dominant negative-acting p85. Interestingly, the 3'-inositol lipid kinase activity of the enzyme appeared to be crucial since the effects of glucose were also reversed by expression of the 3'-phosphatase PTEN (Fig. 5c). Introduction

of PTEN also caused a paradoxical activation of L-PK promoter activity in cells maintained at low glucose, although the mechanism involved in this induction remains obscure. These data also suggest that the production of 3'-phosphorylated lipids, rather than the protein kinase activity of PI3K, is responsible for both the activatory and inhibitory effects of the enzyme. Whether this reflects different pools or species of inositol lipid (*e.g.* phosphatidylinositol 3,4-bisphosphate *versus* PIP<sub>3</sub>) remains to be elucidated.

Our study also demonstrates that stimulation of PI3K activity by elevated glucose concentrations, as recently reported in INS-1  $\beta$ -cells (65), is necessary for the induction of the L-PK gene by insulin. Compatible with the idea that this involves a para/autocrine effect of secreted insulin, Harvey *et al.* (66) have recently demonstrated that added insulin increases PIP<sub>3</sub> levels in the rat CRI-G1 insulinoma cell line, presumably by the activation of PI3K. Similarly, our own studies have demonstrated that either 30 mM glucose or 20 nM insulin increases both PIP<sub>3</sub> levels and type I PI3K activity in MIN6 cells 2–3-fold.<sup>4</sup>

**Role of AMPK**—We recently demonstrated that inhibition of AMPK is a critical event in the regulation of L-PK promoter activity by glucose (52). Thus, activation of AMPK with 5-aminoimidazole-4-carboxamide ribonucleoside, which is converted into the AMP analogue 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (67), leads to the suppression of L-PK gene expression in liver (30, 68) as well as in  $\beta$ -cell-derived HIT-T15, INS-1 (69), and MIN6 (52) cells. Specific inhibition of the  $\alpha$ 2 isoform of AMPK by antibody microinjection fully mimics the effect of high glucose concentrations on L-PK promoter activity in MIN6 cells (52). Incubation of islets with 5-amino-4-imidazolecarboxamide riboside leads to a weak activation of insulin secretion at low glucose concentrations, but then substantially inhibits further increases in insulin release as the glucose concentration is elevated (69, 70). It is therefore possible, but at present not proven, that inhibition of AMPK activity, achieved by the microinjection of antibodies into single cells, may lead to an activation of insulin secretion at low glucose concentrations. The released insulin may then, in turn, bind to insulin receptors and activate PPI and L-PK gene transcription. Consistent with this view, the activation of L-PK promoter activity resulting from the microinjection of anti-AMPK- $\alpha$ 2 antibodies was also sensitive to PI3K blockade (Fig. 7). These observations suggest that AMPK lies either on a signaling pathway upstream of PI3K or on a separate signaling pathway that is responsible for repression of the L-PK gene in low glucose.

This study supports the view that the L-PK promoter may be regulated by insulin through both glucose metabolism-dependent and -independent pathways. The relative importance of these pathways may, however, depend on the cell type and culture conditions. First, as demonstrated in this study on MIN6 cells, insulin can act via a signaling pathway that involves PI3K and presumably a subsequent protein kinase cascade, perhaps involving phospholipid-dependent kinase-1 and protein kinase B/Akt (71). Second, in liver and INS-1  $\beta$ -cells, changes in the intracellular concentration of a glucose metabolite such as Glu-6-P may also activate L-PK gene transcription directly. Thus, 2-deoxyglucose increased the level of L-PK mRNA in INS-1 cells (~1.5-fold increase at 10 mM 2-deoxyglucose) (3), albeit more weakly than glucose (3-fold increase at 10 mM). The poorer efficacy of 2-deoxyglucose compared with glucose in L-PK gene transcription may reflect its inability to

<sup>3</sup> A. Varadi, unpublished data.

<sup>4</sup> I. Rafiq, K. Venkateswarlu, P. Shepherd, and G. A. Rutter, unpublished data.



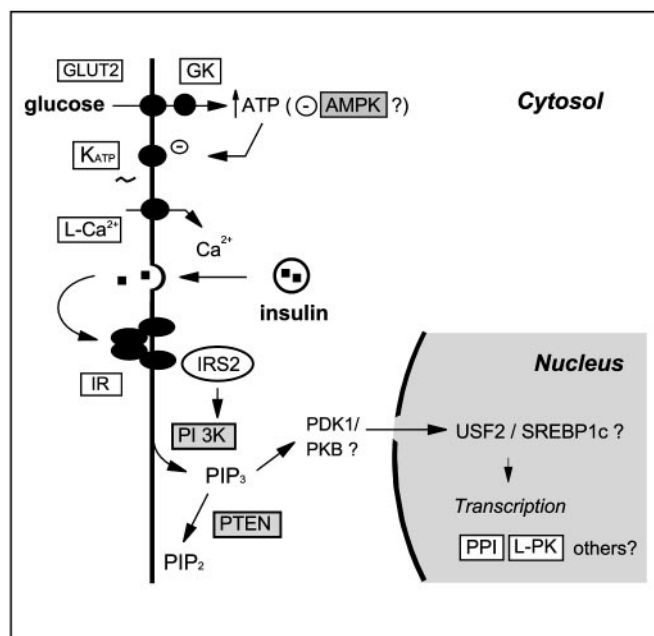


FIG. 8. Scheme illustrating the proposed events underlying the regulation of PPI and L-PK gene transcription by glucose. Uptake of glucose via the glucose transporter GLUT2 and phosphorylation by GK lead to enhanced glycolysis, ATP production, and inhibition of AMPK. Closure of ATP-sensitive  $K^+$  channels ( $K_{ATP}$ ) then provokes cell depolarization and influx of  $Ca^{2+}$  via L-type  $Ca^{2+}$  channels ( $L-Ca^{2+}$ ), triggering the exocytosis of insulin. Secreted insulin then rebinds to the insulin receptor (IR), causing phosphorylation of insulin receptor substrate-2 (IRS2), recruitment of PI3K, and generation of  $PIP_3$ . Subsequent cascades of protein phosphorylation (or dephosphorylation), perhaps involving phospholipid-dependent kinase-1 (PDK1) and protein kinase B (PKB) then lead to changes in the distribution or transactivation capacity of transcription factors such as USF2 or SREBP-1c. Transcription of PPI, L-PK, and other genes then ensues.  $PIP_3$  may be dephosphorylated by the action of PTEN. See "Discussion" for further details.

stimulate insulin secretion efficiently (72). Furthermore, inhibition of PI3K activity and dephosphorylation of 3'-phosphoinositides both blocked the effects of glucose, implicating insulin receptor activation as an early event in the signal transduction pathway leading to transcriptional activation. Importantly, these data also suggest that the PPI and L-PK genes are regulated directly by insulin, and not indirectly as a result of induction of GK and stimulation of glucose phosphorylation. Thus, GK gene expression (Fig. 6a) and glucose metabolism (Fig. 6, b and c) were unaltered by culture with insulin in this model, consistent with the absence of changes in islet GK gene expression in starved *versus* fed animals (32). It therefore seems likely that the increase in L-PK promoter activity (this work) and L-PK mRNA levels (73) may be due to the activation of insulin secretion under these conditions (73).

**Potential Signaling Pathways and Transcription Factors Involved in the Regulation of L-PK Gene Transcription by Glucose and Insulin**—An important question is to define the molecular target of the two potential signaling mechanisms leading to the induction of the L-PK gene. One intriguing possibility is that both pathways converge on a single target molecule, which may be phosphorylated by an insulin-activated protein kinase. Changes in the intracellular levels of Glu-6-P/xylulose 5-phosphate could then interact with this pathway by influencing the phosphorylation state of this target through changes in the activity of a protein phosphatase (such protein phosphatase 2A) (30, 34).

What may be the target of the putative phosphorylation event described above? Both SREBP-1c (74) and USF1/USF2

(26, 27) have been implicated in the regulation by glucose of the L-PK promoter in liver, but their respective roles are at present uncertain (75). Each of these factors binds to oligonucleotides corresponding to the L-PK L4 box, with USF1/2 binding with the greatest avidity (33). In islet  $\beta$ -cells, injection of blocking antibodies demonstrated that USF2 function is essential for L-PK induction by glucose (26). However, a dominant-negative form of USF2 was found to be ineffective in inhibiting L-PK gene induction in INS-1 cells (28) and liver cells (29). We have found that suppression of SREBP-1c function in MIN6 cells with a dominant-negative form of the protein (74) markedly reduces glucose-activation of the promoter,<sup>5</sup> suggesting that this factor is required for the regulation of the gene in  $\beta$ -cells. However, the L-PK promoter appears to be less dependent upon SREBP-1c activity than other glucose-regulated genes in  $\beta$ -cells<sup>6</sup> and liver cells (74–76).

**Conclusion**—These studies demonstrate that both the PPI and L-PK promoters can be regulated by insulin in MIN6  $\beta$ -cells. In this model system, release of insulin and the activation of a PI3K-dependent signaling pathway may be the predominant mechanisms by which glucose activates glucose-sensitive promoters (Fig. 8).

**Acknowledgments**—We thank Alan Leard and Dr. Mark Jepsen (Bristol Medical Research Council Imaging Facility) for assistance with imaging experiments. We thank Dr. C. Zhao for measurements of insulin secretion, Drs. L. Agius and C. B. Newgard for providing GK adenovirus, and Drs. Isabelle Leclerc and Axel Kahn for useful discussions.

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<sup>6</sup> G. da Silva Xavier, C. Andreolas, and G. A. Rutter, unpublished data.

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**Regulation of Gene Expression by Glucose in Pancreatic  $\beta$ -Cells (MIN6) via Insulin Secretion and Activation of Phosphatidylinositol 3'-Kinase**

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*J. Biol. Chem.* 2000, 275:36269-36277.

doi: 10.1074/jbc.M006597200 originally published online August 30, 2000

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Access the most updated version of this article at doi: [10.1074/jbc.M006597200](https://doi.org/10.1074/jbc.M006597200)

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